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Thermostability of Antigens Associated with Serotype of *Rhizobium japonicum*

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The antigens associated with serologically distinct strains of *Rhizobium japonicum* were found to differ in heat sensitivity. Cell preparations from 4 out of 12 strains retained agglutinability, and 1 out of 5 retained antigenicity after they were heated to 120 C. Antigenicity was reduced in most strains after heating to 100 C for 30 min, but agglutinability was not affected by this treatment. This suggests that the antigens are protein-polysaccharide-lipid complexes described for O-type antigens. Cells of strain 46, however, retained both agglutinability and antigenicity after heating to 120 C for 1 hr, and thus a protein in its structure seems improbable. Antigens associated with bacteria from soybean nodules responded to heat treatment in essentially the same manner as those from the same strain grown in nutrient broth. Certain serotypes showed a tendency to agglutinate spontaneously. A heat treatment of 100 C for 30 min, to remove nodule debris and destroy certain blocking antigens, did not interfere with the agglutination reaction. Live cells induced antiserum in rabbit to a higher titer than did heat-treated cells.

The soybean nodule bacterium, *Rhizobium japonicum* (Kirchner) Buchanan, can be separated into a number of serotypically distinct strains on the basis of an agglutination test (3). In previous work, we have shown that bacteria expressed from nodules can be serotyped directly without culturing (7). To improve the accuracy and interpretation of the "quick test," information was needed on the thermostability of the O-type antigens of *R. japonicum*.

The review by Allen and Allen (1) shows that a mass of data is available on the morphological, cultural, and biochemical properties of the rhizobia, but most of it deals with the free-living, culture-grown organisms. The dominant cell type in the nodule, where nitrogen fixation takes place, is commonly called a bacteriod. Some of the differences between the cultivated form and the bacteriod types have been summarized by Jordan (6). Both forms are used in studies made to determine the efficiency of inoculation procedures. Because of their known differences and the scarcity of information on the bacteriod, this study included both the free-living and bacteriod forms. Types of antigens are known to differ in thermal resistance. The Forssman heterophilic antigen can be boiled or autoclaved for several hours without being completely denatured, the O type is known to withstand temperatures of 100 C for at least 2 hr, and the Vi type is destroyed at 100 C in 5 min (2).

Drözańska (4) found two types of thermal resistance in the antigens of nine strains of *R. trifolii*. In one type, the agglutination reaction was inhibited by 1 hr of heating at 120 C, but not at 60 or 100 C. In the other type, the reaction was weaker after heating at 60 C than it was after heating at the higher temperatures. The antiserum was prepared by inoculating rabbits with cells heated to 100 C for 2.5 hr.

Similarly, in serological classification of *R. japonicum* from large numbers of soybean nodules in our laboratory, steamed antigens of some strains agglutinated with more dilute antiserum than did unsteamed antigens of the same strains. With others, agglutinability either remained unchanged or was decreased by the process. Seroagglutination testing of some strains proved difficult because of a marked tendency of the cells to clump and fall out of suspension during preliminary steaming. This paper reports the heat sensitivity of antigens of the major known serotypes of *R. japonicum* in regard to both their antigenicity in rabbits and their agglutinability with homologous antisera.

TABLE 1. *Agglutinability of heat treated Rhizobium japonicum cells, as shown by changes in the indicated titer (× 100) of homologous antisera when used as test antigens*[a]

Strain	Heat treatment of cells used as test antigens							
	Cells from culture				Cells from nodule			
	Unheated	100 C 30 min	120 C 30 min	120 C 120 min	Unheated	100 C 30 min	120 C 30 min	120 C 120 min
3	16	16	1	UC[b]	4	8	UC	UC
31	16	16	2	1	32	16	8	8
38	32	16	8	8	64	64	UC	UC
46	32	32	32	16	2	32	32	32
62	16	16	4	4	32	32	UC	UC
76	32	32	16	16	32	64	32	32
94	8	8	4	4	32	16	4	2
110	16	8	4	2	16	32	16	8
122	64	32	32	8	64	64	UC	UC
123	8	8	16	16	4	8	8	16
125	64	32	16	8	64	64	UC	UC
135	8	8	16	8	8	4	4	8

[a] Antisera induced in rabbits from living culture.

[b] UC = unstable culture indicated by self-agglutination of antigen in control tube.

MATERIALS AND METHODS

Antisera for 12 serotypically distinct strains of *R. japonicum* were prepared by injecting live culture-grown cells into rabbits (9). Other antisera were prepared by using cells grown in nodules. Effect of heat on antigenicity was studied with five strains by comparing the titer of antisera induced in rabbits by portions of the same culture when alive, when heated to 100 C for 30 min, and when heated to 120 C for 1 hr. (Antigenicity, as used here, indicates the ability of *R. japonicum* cells to stimulate antibody formation in rabbits. Agglutinability is used to denote the ability of bacterial cells to agglutinate with homologous antiserum.) Agglutinability was tested by the tube method with antiserum at 1:100 dilution and incubation for 3 hr at 52 C. Titer determinations were made by the double-dilution tube method. Broth cultures were prepared by inoculating cells from a single colony into yeast mannitol broth and allowing them to grow for 7 days, by which time the concentration of cells was approximately 10 million per ml. Suspensions of cells of the same strain were prepared from soybean nodules by the method previously described (7).

For the agglutination tests, cell suspensions from each strain were prepared from culture or from nodules. One portion of each suspension was left unheated; a second portion was heated to 100 C for 30 min; a third portion was heated to 120 C for 30 min; and a fourth portion was heated at 120 C for 120 min. The homologous antiserum was titrated against each lot of treated cells.

Soybean plants grown in sterile sand with nitrogen-free nutrient solution in the greenhouse, and also those grown in a field devoid of *R. japonicum*, were inoculated with 65 single-strain cultures of rhizobia. Nodules induced by each strain were harvested.

Individual portions of the bacterial suspension from each of five nodules were heat-treated and checked for spontaneous agglutination. If the cells remained suspended, they were then tested with homologous antiserum at 1:100 dilution.

RESULTS

Cell preparations of all strains tested, whether grown in culture or expressed from soybean nodules, agglutinated in the presence of homologous antisera almost as well after heating at 100 C for 30 min as they did when unheated (Table 1). In fact, this treatment significantly increased agglutinability for strain 46 cells obtained directly from nodules. More drastic heat treatment reduced agglutinability of several strains and induced self-agglutination in others. Cells of strain 46, 76, 123, and 135, however, suffered no appreciable loss of agglutinability after an exposure to 120 C for 2 hr.

Heat treatment of cells used for inoculation usually lowered the titer of antisera induced (Table 2), although this was less evident when the cells were obtained from nodules. Strain 46, which had shown the most thermal stability in agglutinability, was also the most stable antigen in the rabbit. Cells of strain 110 were the most vulnerable of those tested and retained only slight antigenicity after exposure to 100 C. Antisera induced by four of five strains tested were usually of higher titer when cells from nodules, rather than from cultures, were used as test antigens (Table 2). This was true regardless of the type of cells used in the production of the antiserum. However, cultured cells of strain 46 used as test antigens indicated the homologous antiserum to be of higher titer than cells from nodules.

Strains of the same serotype showed greater similarity in the tendency of cells to agglutinate spontaneously after heating than did those not so related (Table 3). This tendency was increased by the more severe heat treatments. Cells derived from nodules of greenhouse plants grown in sand showed a tendency to retain their stability better than did cells from nodules of field-grown plants. When the field plants were harvested, they were physiologically more mature than those of the greenhouse.

Self-agglutination occurred more frequently with the heated cells obtained from nodules

TABLE 2. *Antigenicity of heat treated Rhizobium japonicum cells as shown by titer (× 100) of antiserum*[a]

Test antigens		Heat treatment of cells inoculated in rabbits							
		Cells from culture				Cells from nodules			
Strain	Source	Unheated	100 C 30 min	120 C 10 min	120 C 60 min	Unheated	100 C 30 min	120 C 10 min	120 C 60 min
46	Culture	64	32	32	32	32	32	—[b]	16
	Nodule	16	16	8	8	8	16	—	16
76	Culture	32	4	—	4	16	4	—	8
	Nodule	32	8	—	8	32	16	—	16
110	Culture	16	2	NR[c]	—	8	2	NR	NR
	Nodule	32	4	NR	—	32	8	2	2
122	Culture	32	8	2	—	16	8	—	—
	Nodule	64	16	2	—	32	32	—	—
125	Culture	32	4	1	NR	16	16	4	1
	Nodule	64	8	1	NR	16	32	32	2

[a] Antiserum was titrated against homologous cells of both cultural and nodular origin pretreated by exposure to 100 C for 30 min.
[b] Tests not made.
[c] NR = no reaction detected.

than with those from culture. It was necessary to heat-treat several preparations of some strains for the agglutination tests (Table 1) in order to find one in which the cells remained suspended after the more extreme applications. In 5 of the 12 strains, this was never achieved for cells from the nodules. In marked contrast, cultivated cells of all strains withstood 120 C for 10 min, and all except those of strain 3 withstood 120 C for 2 hr.

TABLE 3. *Frequency of self-agglutination in nodule suspensions of different serotypes*

Serotype	No. of strains tested[a]	Nodule suspensions that self-agglutinated (%)			
		Heated 100 C, 30 min		Heated 120 C, 30 min	
		Greenhouse[b]	Field[b]	Greenhouse	Field
3	16	52	82	100	100
31	7	0	0	0	24
38	9	0	18	100	100
46	9	0	11	0	34
62	3	0	33	14	100
76	1	0	0	0	0
94	1	0	0	0	0
110	6	0	31	79	94
122	2	0	0	50	100
123	8	3	5	26	59
125	2	0	0	100	100
135	1	0	0	0	0

[a] Five individual nodules tested for each strain.
[b] Greenhouse—nodules grown on plants in sterile sand. Field—nodules from inoculated plants grown in field free of other *Rhizobium japonicum* strains.

DISCUSSION

Two types of response to heat treatment were noted among 12 *R. japonicum* strains of different serotypes. The apparent changes in titer of homologous antisera reflected a change in the agglutinability of the heat-treated cells used as test antigens. Therefore, the most stable strain lost little agglutinability after cells were heated to 120 C for 2 hr. The most labile, however, required very high concentrations of homologous antiserum after exposure to the same temperature for only 30 min (strains 46 and 3, Table 1). The other strains were intermediate.

Fundamental differences in chemical structure of antigens may be indicated by the manner in which bacteria of different serotypes resisted heat damage. Strain 46 induced antiserum of approximately the same titer after the cells had been heated to 120 C for 1 hr as when the cells were unheated. This suggested a polysaccharide structure. In contrast, antigens associated with strain 110 lost most of their ability to induce antibody in rabbit, but retained their ability to agglutinate with homologous antisera after treatment at 100 C (Table 2). This reaction implies the protein-polysaccharide-lipid complex described by Salton (8) for O antigens. This theory assumes that heat-labile protein fraction confers antigenicity, but the more heat-resistant polysaccharides determine specificity.

Dudman (5), by using immune diffusion techniques for analysis of two strains of *R. meliloti*, found three groups of precipitin bands, two of which were characterized as polysaccharides and the third as a protein.

Bacterial cells from soybean nodules are similar morphologically to those grown in culture, but they agglutinate more readily and their antigens are somewhat more resistant to heating. This suggests chemical or physical differences in the antigens from these two sources, but these differences are not great enough to interfere with successful serotyping of nodule suspensions with antiserum produced from bacteria grown in culture.

The bacterial suspensions from nodules tended to self-agglutinate on heating more than did those from laboratory cultures. This was often avoided by using less mature nodules. Some serotypes are more prone to self-agglutinate than are others. Strains which produce abundant gum in culture were more likely to self-agglutinate; however, these strains also tended to self-agglutinate when cells were taken from nodules where gum production is not evident.

When suspensions of cells from nodules were used for rabbit inoculation, the antisera induced agglutinated cultured cells of the same strain that had instigated the nodule. Furthermore, the number and strength of cross-reactions, compared with antiserum induced by culture-grown cells of the same strain, were not increased significantly. This indicates that the traces of plant antigens are not likely to account for the minor differences found between the culture-grown and nodule-derived bacteria.

On the basis of these results and after several years of experience in serotyping *R. japonicum* directly from soybean nodules, we recommend Vincent's procedure for producing antiserum with pure-culture live bacteria for injection of the rabbits (9).

For routine agglutination tests, we use bacteria from a single nodule suspended in physiological saline (7). A preliminary heat-treatment of 100 C for 30 min tends to precipitate nodule tissue and makes agglutination reactions easier to read; in most cases, this does not materially affect agglutination. The increased agglutinability of heated cells of strain 46 derived from nodules suggests that certain heat-labile blocking antigens may be destroyed by the treatment.

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